

increased the sensitivity of JFH1 virus to antibody neutralization [Grove et al., 2008].

In conclusion, the present study using J6/JFH1 virus, another HCVcc strain, has demonstrated that the N534H mutation within the sixth *N*-glycosylation site of the E2 glycoprotein, and the T416A mutation near the first *N*-glycosylation site to a lesser extent, markedly enhances sensitivity to neutralization by antibodies in sera of HCV-infected patients. These results suggest that glycans on Asn-534 of the HCV E2 glycoprotein plays an important role in protecting the virus from humoral immune mechanisms of the host.

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Molecular mechanism of hepatitis C virus-induced glucose metabolic disorders

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Hepatitis C virus (HCV) infection causes not only intrahepatic diseases but also extrahepatic manifestations, including metabolic disorders. Chronic HCV infection is often associated with type 2 diabetes. However, the precise mechanism underlying this association is still unclear. Glucose is transported into hepatocytes via glucose transporter 2 (GLUT2). Hepatocytes play a crucial role in maintaining plasma glucose homeostasis via the gluconeogenic and glycolytic pathways. We have been investigating the molecular mechanism of HCV-related type 2 diabetes using HCV RNA replicon cells and HCV J6/JFH1 system. We found that HCV replication down-regulates cell surface expression of GLUT2 at the transcriptional level. We also found that HCV infection promotes hepatic gluconeogenesis in HCV J6/JFH1-infected Huh-7.5 cells. HCV infection transcriptionally up-regulated the genes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), the rate-limiting enzymes for hepatic gluconeogenesis. Gene expression of PEPCK and G6Pase was regulated by the transcription factor forkhead box O1 (FoxO1) in HCV-infected cells. Phosphorylation of FoxO1 at Ser319 was markedly diminished in HCV-infected cells, resulting in increased nuclear accumulation of FoxO1. HCV NS5A protein was directly linked with the FoxO1-dependent increased gluconeogenesis. This paper will discuss the current model of HCV-induced glucose metabolic disorders.

Keywords: HCV, diabetes, gluconeogenesis, GLUT2, FoxO1, JNK, NS5A

INTRODUCTION

Hepatitis C virus (HCV) is a positive-sense, single stranded RNA virus that belongs to the genus *Hepacivirus* of the family *Flaviviridae*. The approximately 9.6-kb HCV genome encodes a unique open reading frame that is translated into a polyprotein of about 3,000 amino acids, which is cleaved by cellular signalases and viral proteases to generate at least 10 viral proteins, such as core, envelope 1 (E1) and E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Choo et al., 1991; Lemon et al., 2007).

Hepatitis C virus is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. More than 170 million people worldwide are chronically infected with HCV (Poynard et al., 2003). Persistent HCV infection causes not only liver diseases but also extrahepatic manifestations. It is well established that HCV perturbs the glucose metabolism, leading to insulin resistance and type 2 diabetes in predisposed individuals. Several epidemiological, clinical, and experimental data suggested that HCV infection serves as an additional risk factor for the development of diabetes (Mason et al., 1999; Negro and Alaei, 2009; Negro, 2011). HCV-related glucose metabolic changes and insulin resistance and diabetes have significant clinical consequences, such as accelerated fibrogenesis, increased incidence of hepatocellular carcinoma, and reduced virological response to interferon (IFN)- α -based therapy (Negro, 2011). Therefore, it is very important to clarify the molecular mechanism of HCV-related diabetes. However, the precise mechanisms are poorly understood.

Experimental data suggest a direct interference of HCV with the insulin signaling pathway. Transgenic mice expressing HCV

core gene exhibit insulin resistance (Shintani et al., 2004; Koike, 2007). In this transgenic mice model, both tyrosine phosphorylation of the insulin receptor substrate (IRS)-1 and IRS-2 are decreased. These decreases are recovered when the proteasome activator PA28 γ is deleted, suggesting that the HCV core protein suppresses insulin signaling through a PA28 γ -dependent pathway (Miyamoto et al., 2007). Several other reports also showed a link of the HCV core protein with insulin resistance (Kawaguchi et al., 2004; Paziienza et al., 2007).

Hepatocytes play a crucial role in maintaining plasma glucose homeostasis by adjusting the balance between hepatic glucose production and utilization via the gluconeogenic and glycolytic pathways, respectively. Gluconeogenesis is mainly regulated at the transcriptional level of the glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) genes, whereas glycolysis is mainly regulated by glucokinase (GK). Gluconeogenesis and glycolysis are coordinated so that one pathway is highly active within a cell while the other is relatively inactive. It is well known that increased hepatic glucose production via gluconeogenesis is a major feature of type 2 diabetes (Clore et al., 2000).

To identify a novel mechanism of HCV-related diabetes, we have been investigating the effects of HCV on glucose production in hepatocytes using HCV RNA replicon cells (Lohmann et al., 1999) and HCV J6/JFH1 cell culture system (Lindenbach et al., 2005; Wakita et al., 2005; Bungyoku et al., 2009). We previously reported that HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporter 2 (GLUT2; Kasai et al., 2009). Furthermore, we

recently reported that HCV promotes hepatic gluconeogenesis via an NS5A-mediated, forkhead box O1 (FoxO1)-dependent pathway, resulting in increased cellular glucose production in hepatocytes (Deng et al., 2011). This paper discusses our current model for HCV-induced glucose metabolic disorders.

HCV REPLICATION DOWN-REGULATES CELL SURFACE EXPRESSION OF GLUT2

The uptake of glucose into cells is conducted by the facilitative glucose carrier, glucose transporters (GLUTs). GLUTs are integral membrane proteins that contain 12 membrane-spanning helices. To date, a total of 14 isoforms have been identified in the GLUT family (Wu and Freeze, 2002; Macheda et al., 2005; Godoy et al., 2006). Glucose is transported into hepatocytes by GLUT2. We previously reported that HCV J6/JFH1 infection suppresses hepatocytic glucose uptake through down-regulation of surface expression of GLUT2 in human hepatoma cell line, Huh-7.5 cells (Kasai et al., 2009). We also demonstrated that GLUT2 expression in hepatocytes of the liver tissues from HCV-infected patients was significantly lower than in those from patients without HCV infection. Our data suggest that HCV infection down-regulates GLUT2 expression at transcriptional level. We are currently analyzing transcriptional control of human GLUT2 promoter in HCV replicon cells as well as in HCV J6/JFH1-infected cells.

HCV INFECTION PROMOTES HEPATIC GLUCONEOGENESIS

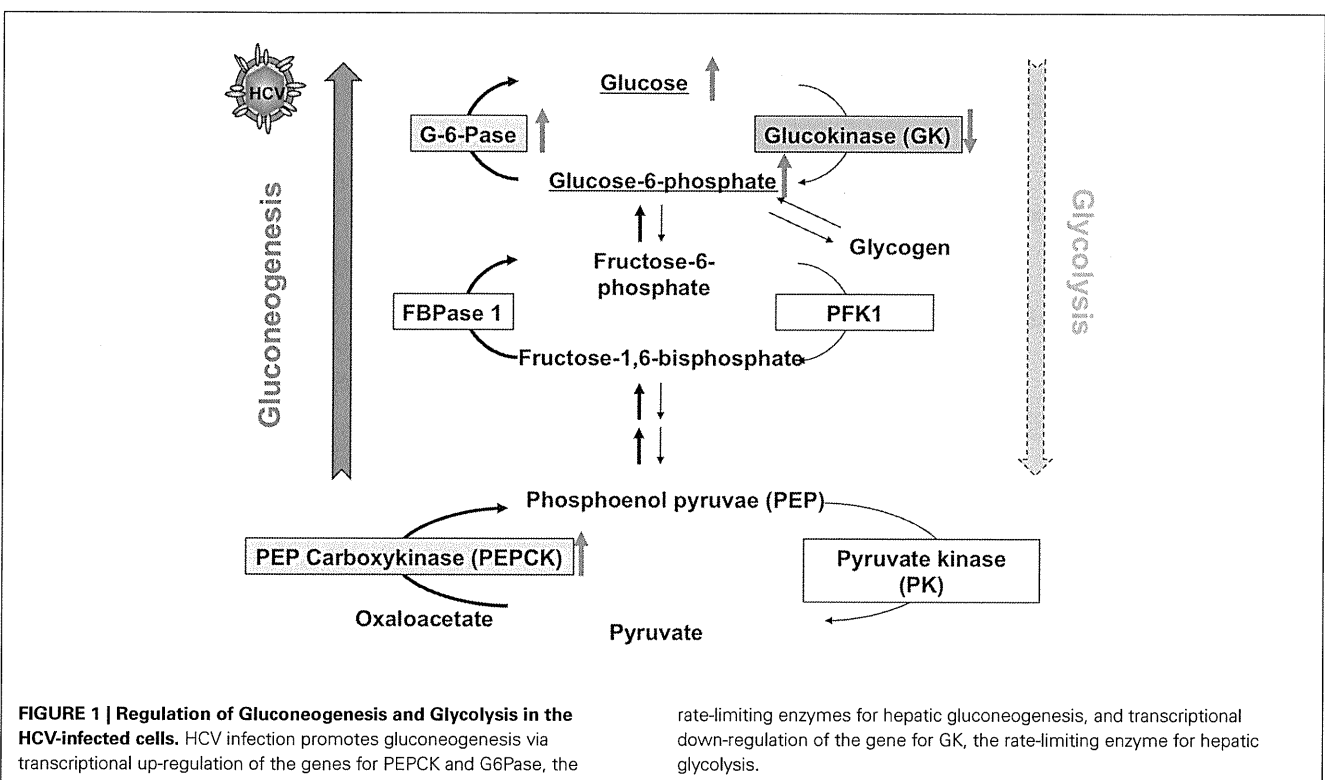
Then we analyzed hepatic glucose production and expression of transcription factors using HCV replicon cells and HCVcc system in order to clarify a role of HCV infection in glucose metabolic changes. Hepatic glucose production is usually regulated by

gluconeogenesis and glycolysis. Therefore, we examined whether HCV infection induces gluconeogenesis or glycolysis. We found that the PEPCK and G6Pase genes were transcriptionally up-regulated in J6/JFH1-infected cells (Figure 1). On the other hand, the GK gene was transcriptionally down-regulated in HCV-infected cells. We obtained similar data in HCV replicon cells (both in subgenomic replicon cells and full-genomic replicon cells). When HCV replication was suppressed by IFN treatment, the up-regulation of PEPCK and G6Pase gene expression as well as the down-regulation of GK gene expression were canceled. From these results, HCV infection selectively up-regulates PEPCK and G6Pase genes, whereas HCV infection down-regulates GK gene (Deng et al., 2011).

Both HCV replicon cells and HCV-infected cells produced greater amounts of glucose than the control cells. IFN treatment canceled the enhanced glucose production in HCV replicon cells as well as in HCV-infected cells. G6P is an important precursor molecule that is converted to glucose in the gluconeogenesis pathway (Figure 1). Our metabolite analysis showed that a significantly higher level of G6P was accumulated in HCV-infected cells than in the control cells, suggesting that HCV indeed promotes hepatic gluconeogenesis to cause hyperglycemia. There is a trend toward an increase in gluconeogenesis in HCV-infected cells (Figure 1).

HCV SUPPRESSES FoxO1 PHOSPHORYLATION AT Ser319, LEADING TO THE NUCLEAR ACCUMULATION OF FoxO1

It has been reported that G6Pase, PEPCK, and GK are regulated by certain transcription factors, including FoxO1 (Hirota et al., 2008), hepatic nuclear factor 4 α (HNF-4 α ; Hirota et al.,



2008), Krüppel-like factor 15 (KLF15; Takashima et al., 2010), and cyclic AMP (cAMP) response element binding protein (CREB; Rozance et al., 2008). While we were analyzing these factors in both HCV replicon cells and HCV J6/JFH1-infected cells, we found the involvement of the FoxO1 in the transcriptional activation of G6Pase and PEPCK (Deng et al., 2011). It is known that the FoxO1 enhances gluconeogenesis through the transcriptional activation of various genes, including G6Pase and PEPCK (Gross et al., 2008). The function of FoxO1 is regulated by post-translational modifications, including phosphorylation, ubiquitylation, and acetylation (Tzivion et al., 2011). The phosphorylated form of FoxO1 is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (Figure 2). Phosphorylation status of FoxO1 at Ser319 is critical for FoxO1 nuclear exclusion (Zhao et al., 2004). Although the total amounts of FoxO1 protein were unchanged, FoxO1 phosphorylation at Ser319 was markedly suppressed in HCV-infected cells compared to that in the mock-infected cells. It is known that the FoxO1 is phosphorylated by the protein kinase Akt and is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (Tzivion et al., 2011). The majority of FoxO1 was accumulated in the nuclear fraction in HCV-infected cells, whereas in control cells FoxO1 was distributed in both the nuclear and cytoplasmic fractions. Akt phosphorylation was enhanced in HCV-infected cells, although the protein levels of total Akt protein were comparable, which is consistent with the report by Burdette et al. (2010). Our findings suggest an interesting scenario in which the HCV-mediated suppression in FoxO1 phosphorylation is caused by an unknown mechanism independent of Akt activity.

HCV-INDUCED JNK ACTIVATION IS INVOLVED IN THE SUPPRESSION OF FoxO1 PHOSPHORYLATION

It is known that the stress-sensitive serine/threonine kinase JNK regulates FoxO at multiple levels (van der Horst and Burgering, 2007; Karpac and Jasper, 2009). We demonstrated that HCV infection induces phosphorylation and activation of JNK in a time-dependent manner, which is similar to that observed for the suppression of FoxO1 phosphorylation. As a result, c-Jun, a key substrate for JNK, got phosphorylated and activated in HCV-infected cells. The JNK inhibitor SP600125 clearly prevented the phosphorylation of c-Jun, and concomitantly recovered the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that HCV activates the JNK/c-Jun signaling pathway, resulting in the nuclear accumulation of FoxO1 by reducing its phosphorylation status. The detailed mechanisms of HCV-induced suppression of FoxO1 phosphorylation via the JNK/c-Jun signaling pathway remain to be explored. There are at least two possibilities. The JNK/c-Jun signaling pathway (1) suppresses a protein kinase, or (2) activates a protein phosphatase to reduce phosphorylation of FoxO1.

HCV-INDUCED MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION IS INVOLVED IN INCREASED GLUCOSE PRODUCTION THROUGH JNK ACTIVATION

Hepatitis C virus infection increases mitochondrial reactive oxygen species (ROS) production (Deng et al., 2008). *N*-acetyl cysteine (NAC; a general antioxidant) clearly prevented the phosphorylation of JNK, and concomitantly canceled the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that

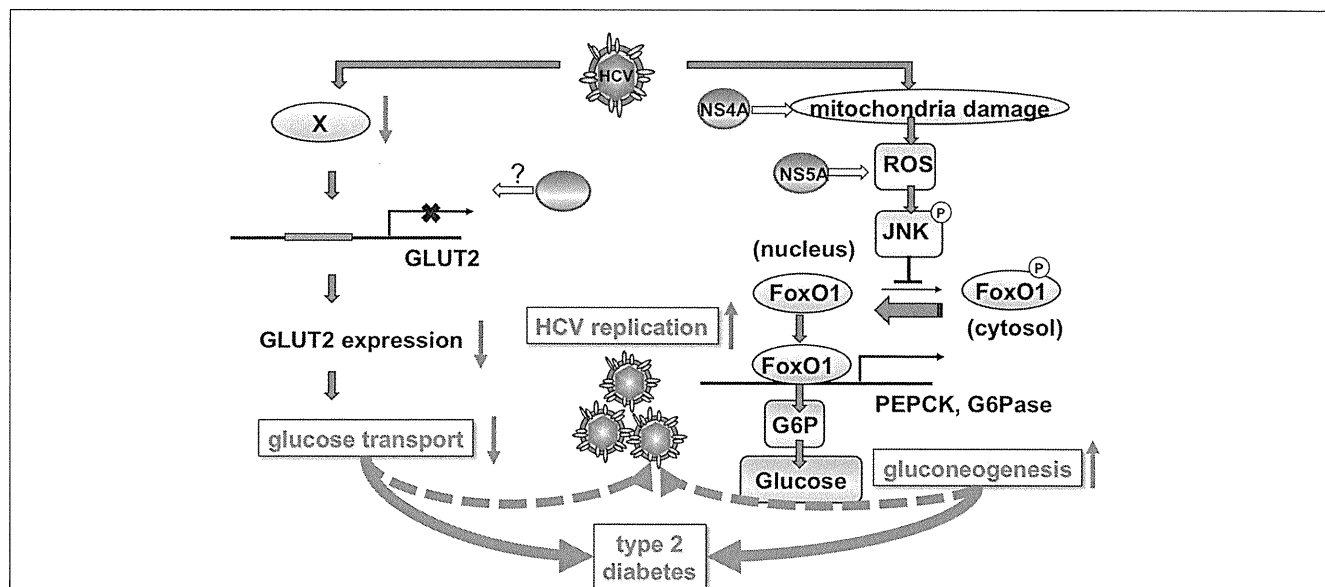


FIGURE 2 | A proposed mechanism of HCV-induced glucose metabolic disorders. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. HCV infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage. HCV

NS5A protein is involved in ROS production. HCV-induced ROS production causes JNK activation, resulting in the decreased phosphorylation and nuclear accumulation of FoxO1. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis. High glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

HCV-induced ROS production is involved in the JNK activation. There was no significant difference in HCV RNA replication or infectious virus release between SP600125- or NAC-treated HCV-infected cells and non-treated HCV-infected cells. These results suggest that ROS-mediated JNK activation plays a key role in the suppression of FoxO1 phosphorylation, nuclear accumulation of FoxO1, and enhancement of glucose production in HCV-infected cells (Deng et al., 2011).

HCV NS5A IS INVOLVED IN THE ENHANCEMENT OF GLUCOSE PRODUCTION

Then we sought to determine which HCV protein(s) is involved in the enhancement of glucose production. Transient expression of NS5A protein in Huh-7.5 cells significantly promoted the gene expression levels of G6Pase and PEPCK determined by real time quantitative RT-PCR. Promoter assay revealed that the level of PEPCK promoter activity was significantly higher in NS5A-expressing cells than in the control cells. Our results suggest that NS5A activate both the PEPCK promoter and the G6Pase promoter, leading to an increase in glucose production (Deng et al., 2011). The study by Banerjee et al. (2010) suggests that the HCV core protein modulates FoxO1 and FoxA2 activation and affects insulin-induced metabolic gene regulation in human hepatocytes. Our results, however, suggest that the HCV core protein is not significantly involved in the increased gluconeogenesis (Deng et al., 2011). The difference between these two studies needs to be explored.

There were previous reports suggesting that ROS production is induced in NS5A-expressing cells (Dionisio et al., 2009) or in hepatocytes of NS5A transgenic mice (Wang et al., 2009). We therefore sought to determine whether NS5A contributes to increased hepatic gluconeogenesis through the induction of ROS production. NS5A-expressing cells displayed a much stronger signal of ROS than in control cells. NS5A-expressing cells promoted phosphorylation level at Ser63 of c-Jun and suppressed FoxO1 phosphorylation at Ser319, suggesting that NS5A mediates JNK/c-Jun activation and FoxO1 phosphorylation suppression. These results suggest that NS5A play a role in the HCV-induced enhancement of hepatic gluconeogenesis through JNK/c-Jun activation and FoxO1 phosphorylation suppression.

CONCLUSION AND FUTURE PERSPECTIVES

Taken together, we propose a model of HCV-induced glucose metabolic disorders as shown in **Figure 2**. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. As GLUT2 is a facilitative GLUT, it ensures large bidirectional fluxes of glucose in and out the cell due to its low affinity and high capacity (Leturque et al., 2009). Down-regulated

cell surface expression of GLUT2 results in disruption of bidirectional transport of glucose in hepatocytes. Even in the fasting state, down-regulation of GLUT2 may result in low glucose uptake of hepatocytes, causing hyperglycemia. In the fed state, glucose secretion from hepatocytes may be suppressed due to low level cell surface expression of GLUT2, as GLUT2 is a bidirectional transporter.

Hepatitis C virus infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage (Nomura-Takigawa et al., 2006). HCV NS5A protein is involved in ROS production (Dionisio et al., 2009; Wang et al., 2009; Deng et al., 2011). HCV-induced ROS production causes JNK activation, which results in the decreased phosphorylation and nuclear accumulation of FoxO1 by an unidentified mechanism. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis (Deng et al., 2011).

These two pathways, HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis, may contribute to development of type 2 diabetes in HCV-infected patients at least to some extent. HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis may result in high concentration of glucose in HCV-infected hepatocytes. As suggested in a recent study, low glucose concentration in the hepatocytes inhibits HCV replication (Nakashima et al., 2011). Therefore, high glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

Our understanding of HCV-induced glucose metabolic disorders will require much more work to fully unfold this pathway. Further investigation including the mechanism of HCV-induced GLUT2 downregulation, JNK-mediated decreased phosphorylation of FoxO1, and the possible effect(s) of the dysregulation of hepatic gluconeogenesis on the HCV life cycle and host cells are currently under way.

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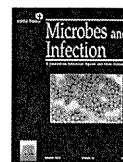


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Original article

Generation of a recombinant reporter hepatitis C virus useful for the analyses of virus entry, intra-cellular replication and virion production

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Abstract

The lack of a culture system that efficiently produces progeny virus has hampered hepatitis C virus (HCV) research. Recently, the discovery of a novel HCV isolate JFH1 and its chimeric derivative J6/JFH1 has led to the development of an efficient virus productive culture system. To construct an easy monitoring system for the viral life cycle of HCV, we generated bicistronic luciferase reporter virus genomes based on the JFH1 and J6/JFH1 isolates, respectively. Transfection of the J6/JFH1-based reporter genome to Huh7.5 cells produced significantly greater levels of progeny virus than transfection of the JFH1 genome. Furthermore, the expression of dominant-negative Vps4, a key molecule of the endosomal sorting complex required for transport machinery, inhibited the virus production of JFH1, but not that of J6/JFH1. These results may account for the different abilities to produce progeny virus between JFH1 and J6/JFH1. Using the J6/JFH1/Luc system, we showed that the two polyanions heparin and polyvinyl sulfate decreased the infectivity of J6/JFH1/Luc virus in a dose-dependent manner. We also analyzed the function of microRNA on HCV replication and found that miR-34b could affect the replication of HCV. The reporter virus generated in this study will be useful for investigating the nature of the HCV life cycle and for identification of HCV inhibitors.

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Keywords: HCV; Reporter virus; Virus production; ESCRT; microRNA

1. Introduction

Hepatitis C virus (HCV) is an enveloped virus and has a positive-stranded RNA genome of about 9.6 kb [1,2]. HCV persistently infects hepatocytes, and the persistent infection can lead to liver cirrhosis and hepatocellular carcinoma. Considering that approximately 170 million people are infected with HCV worldwide [3], HCV is a major public health problem throughout the world. A combination therapy of pegylated interferon- α and ribavirin has been established as the standard of care for treating HCV infection [3,4].

Nonetheless, approximately 50% of individuals with chronic HCV infection are still unable to resolve infection [4,5]. For this reason, more effective therapies are greatly needed against the disease caused by HCV infection [6].

The HCV genome encodes a 3000 amino acid polyprotein which is cleaved by host and viral proteases to yield the mature structural proteins, composed of core and glycoprotein E1 and E2, and the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [1–3]. Translation of the HCV open reading frames is mediated via the 5' untranslated region and a part of the core coding region carrying the internal ribosome entry site (IRES) [1,7].

In 1999, Bartenschlager and his colleagues produced the HCV replicon system, a tissue culture system that recapitulated the RNA replication of HCV in a human hepatoma cell line [8]. In the initial subgenomic replicon system, genes

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unessential for RNA replication that contained the core, E1, E2, p7 and NS2 of the HCV genome were replaced with a genetic cassette carrying an antibiotics resistance gene and IRES from encephalomyocarditis virus (EMCV). The development of a subgenomic replicon system became a driving force for the studies on the mechanism of HCV replication, and these studies revealed numerous biological features of HCV replication. However, the resulting systems were unable to produce progeny virus. Therefore, the nature of the HCV, i.e., the virus production and virus entry, remained unclear for a long while.

Wakita and his colleagues isolated a full-length HCV genome from the sera of a patient with fulminant hepatitis [9]. The HCV strain, designated JFH1, belongs to genotype 2a. The transfection of the Huh7 hepatoma cell line with the JFH1 genome yields a progeny virus called HCVcc that is infectious both *in vivo* and *in vitro*. The HCVcc system allowed us to perform virological studies to investigate the nature of HCV [9,10]. However, the analyses using HCVcc have not been suitable for carrying out high-throughput screening due to the labor-intensive quantitative reverse transcription-PCR methods used in screening and the difficulties presented by the low signal-to-noise ratios.

In this study, to develop a robust tool for use in the screening of HCV replication, we have constructed a genome-length luciferase reporter HCV derived from the JFH1 and J6/JFH1 strains, and used it to analyze the intra-cellular RNA replication and extra-cellular progeny virus production. We demonstrated here that our recombinant reporter HCV system was useful for studying viral genome replication, virus entry, and virion production of HCV.

2. Materials and methods

2.1. Plasmids

The plasmid pFGR-JFH1/Luc, which encodes bicistronic constructs of HCV IRES-driven firefly luciferase reporter genes and the EMCV IRES-driven full-genomic JFH1 genome, was constructed by insertion of the JFH1 full genome of pJFH1 [9] into pSGR-JFH1 [11]. The plasmid pFL-J6/JFH1, which contains a chimeric full-genome composed of the 5'NCR to NS2 region derived from J6 and NS3 to the 3'NCR region from JFH1 [10], was kindly supplied by C.M. Rice of the Center for the Study of Hepatitis C, Rockefeller University. To yield the bicistronic luciferase reporter construct composed of full-length J6/JFH1, the JFH1 full genome of pFGR-JFH1/Luc was replaced with the J6/JFH1 full genome of pFL-J6/JFH1 by digestion with BstZ171, and the resultant plasmid was designated as pFGR-J6/JFH1/Luc. As a negative control for the HCV replication, a non-synonymous mutation at NS5B (GDD to GND), which disrupts NS5B polymerase activity, was introduced into the pFGR-J6/JFH1/Luc NS5B region by site-directed mutagenesis, and the resultant plasmid was designated pFGR-J6/JFH1/Luc (GND).

2.2. Cell culture and indirect immunofluorescence

All experiments described in this study were performed by using Huh7.5 human hepatoma cells, a highly HCV-susceptible subclone of Huh7 cells. The cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 0.01% streptomycin, and were subcultured twice weekly. Huh7.5 cells electroporated with JFH1/Luc or J6/JFH1/Luc RNA were subjected to indirect immunofluorescence analysis as previously reported [12]. The primary antibody used was derived from an HCV-infected patient's serum. The secondary antibody used was fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (MBL, Nagoya, Japan).

2.3. *In vitro* transcription and electroporation

Plasmid DNA was linearized with XbaI, extracted with phenol and chloroform, precipitated with ethanol, and dissolved in RNase-free water. The purified DNA was used for *in vitro* RNA transcription using a T7 Megascript kit (Ambion, Austin, TX) following the manufacturer's protocols. The concentration was determined by measurement of the optical density at 260 nm, and the RNA integrity was checked by agarose gel electrophoresis. The *in vitro*-transcribed RNA (10 µg) was transfected into Huh7.5 cells by means of electroporation (975 µF, 270 V) using a Gene Pulser (Bio-Rad, Hercules, CA). The cells were then cultured in complete medium. The culture fluid of transfected cells was harvested and cleared by passing through 0.45-µm-pore-size filters and stored at -80 °C until use.

2.4. Luciferase assay

The firefly luciferase activity was measured by a luciferase assay system (Promega, Madison, WI). The cells were harvested, washed twice with dication-free phosphate buffered saline (PBS), and lysed in a passive lysis buffer supplied by the manufacturer. A 20-µl sample of the lysate was subjected to a luciferase assay. The luminescence was measured at 10 s after an initial 2 s delay according to the manufacturer's instructions, using a Lumat LB9501 luminometer (Berthold, Freiburg, Germany). The assays were performed in duplicate at least three times, and the mean and standard error were computed.

2.5. Vectors of ESCRT family proteins and DNA transfection

The cDNA of the endosomal sorting complex required for transport (ESCRT) family proteins was amplified from Huh7.5 cells by RT-PCR and cloned into pcDNA3.1-FLAG [13], an expression vector containing a CMV promoter and FLAG tag sequence in pcDNA3.1 (Invitrogen, Carlsbad, CA). For the expression of each ESCRT family protein, Huh7.5 cells were transfected with each ESCRT expression vector by using TransIT LT1 transfection reagents (Takara, Kyoto, Japan). The expression levels of the three ESCRT family proteins in

transfected Huh7.5 cells were monitored by immunoblotting using the anti-FLAG antibody (Sigma–Aldrich, St. Louis, MO).

2.6. Quantification of HCV core protein

HCV core protein in the cells or cell-culture supernatants was quantified by using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA kit; Ortho Clinical Diagnostics). To determine the intra-cellular amounts of core, cell lysates were prepared as described by Schaller et al. [14].

2.7. Blocking of virus attachment and entry with anti-CD81 antibody

Blocking of virus attachment and entry with anti-CD81 antibody was performed essentially as described previously [9]. Huh7.5 cells (6×10^4 cells/well of a 24-well plate) were pre-treated with anti-CD81 antibody (clone JS-81; BD Biosciences) or an isotype-matched control antibody (purified mouse IgG1, isotype control; BD Biosciences) as indicated for 1 h. Cells were then infected with the reporter viruses for 6 h. The viruses were removed, and then the culture medium was replaced with complete DMEM. On day 2 post-infection, the cells were lysed with a passive lysis buffer as mentioned above. The efficiency of infection was monitored by measuring the luciferase activity of the cell lysate.

2.8. Transfection of microRNA inhibitor

Huh7.5 cells were electroporated with luciferase reporter HCV RNA as mentioned above, and then the cells were seeded in a well of a 24-well plate. To analyze the effect of inhibition of microRNA (miR), both a specific miRNA inhibitor (Anti-miRTM miRNA) and a non-targeting negative control (Anti-miRTM miRNA Inhibitors—Negative Control) were purchased from Ambion, Inc. 50 pmol of a specific miRNA inhibitor or negative control were transfected into luciferase reporter RNA-electroporated Huh7.5 cells by using a siPORTTM NeoFXTM Transfection Agent (Ambion) according to the manufacturer's instructions. At 48 h post-transfection, the cells were harvested, and viral replication was determined by luciferase assay of the cell lysate.

2.9. Polyions

The polyanions heparin (mol. wt. 3000), dextran sulfate (mol. wt. 50,000) and polyvinyl sulfate (mol. wt. 150,000), and the polycations polybrene (mol. wt. 3000), DEAE-dextran (mol. wt. 100,000), and poly-L-lysine (mol. wt. 500,000) (all purchased from Sigma) were dissolved in PBS.

3. Results

3.1. Construction and characterization of luciferase reporter HCV

To construct a reporter HCV that can permit easy monitoring of both virus production and intra-cellular viral growth

kinetics, we constructed the bicistronic HCV constructs by inserting a luciferase reporter gene into the 5' end of the coding sequence of the JFH1 or J6/JFH1 full-genome plasmids clone as shown in Fig. 1A. In the transcript derived from bicistronic reporter HCV clone, the HCV and EMCV IRESs are responsible for the translation of the luciferase protein and all HCV proteins, respectively. A reporter construct with NS5B GDD to GND mutation, which disrupts viral polymerase function, was also constructed by site-directed mutagenesis, and served as a negative control for viral genome replication. To examine the replication level of reporter HCVs, we prepared the RNAs from each construct by *in vitro* transcription, and then transfected them into Huh7.5 cells by an electroporation technique. The viral replication was quantified up to 10 days post-transfection by using an HCV core-specific ELISA and luciferase reporter assay. As shown in Fig. 1B, the transfection of RNAs of both the JFH1/Luc and J6/JFH1/Luc reporter clones induced intra-cellular HCV core protein expression, which peaked on day 2 post-transfection. Both JFH1/Luc and J6/JFH1/Luc showed similar kinetics, and the high level core protein expression continued until day 10 post-transfection. As expected, the GND mutant exhibited 100-fold lower intra-cellular core protein expression on day 2 post-transfection. The level of core expression by the GND mutant continued to decline thereafter, and fell below the detection limit on day 10 post-transfection. As shown in Fig. 1C, both JFH1/Luc and J6/JFH1/Luc induced similar levels of luciferase activity in Huh7.5 cells at 4 h after electroporation. This result indicated that both RNAs were electroporated with similar efficiency because RNA replication had not started at that time and all the luciferase was translated from the input RNA. At 4 days post-electroporation, the luciferase activities of both JFH1/Luc and J6/JFH1/Luc were 10-fold greater than those measured at 4 h after electroporation. Subsequently, JFH1/Luc and J6/JFH1/Luc showed almost the same kinetics of luciferase activity until 10 days post-transfection. At 3 days post-electroporation, both JFH1/Luc and J6/JFH1/Luc electroporated cells were stained with HCV-positive patient sera, and the rate of intra-cellular replication was then visualized using immunofluorescent microscopy as previously reported [12]. As a result, the HCV-positive rates were 17% and 19% for JFH1/Luc and J6/JFH1/Luc, respectively (Fig. 1D). These results indicated that the luciferase activity of reporter HCV-transfected cells reflected the intra-cellular viral replication, and also suggested that both JFH1 and J6/JFH1 had similar intra-cellular replication ability in Huh7.5 cells.

3.2. Production of cell-free infectious progeny virions in luciferase reporter HCV RNA-transfected cells

Next, we assessed the potential of the reporter HCV to produce infectious progeny virions. Huh7.5 cells were electroporated with the reporter RNAs, and the culture supernatant was collected at various time points. To analyze the release of progeny virions from the reporter RNA-electroporated cells, the amounts of core protein in culture supernatants were

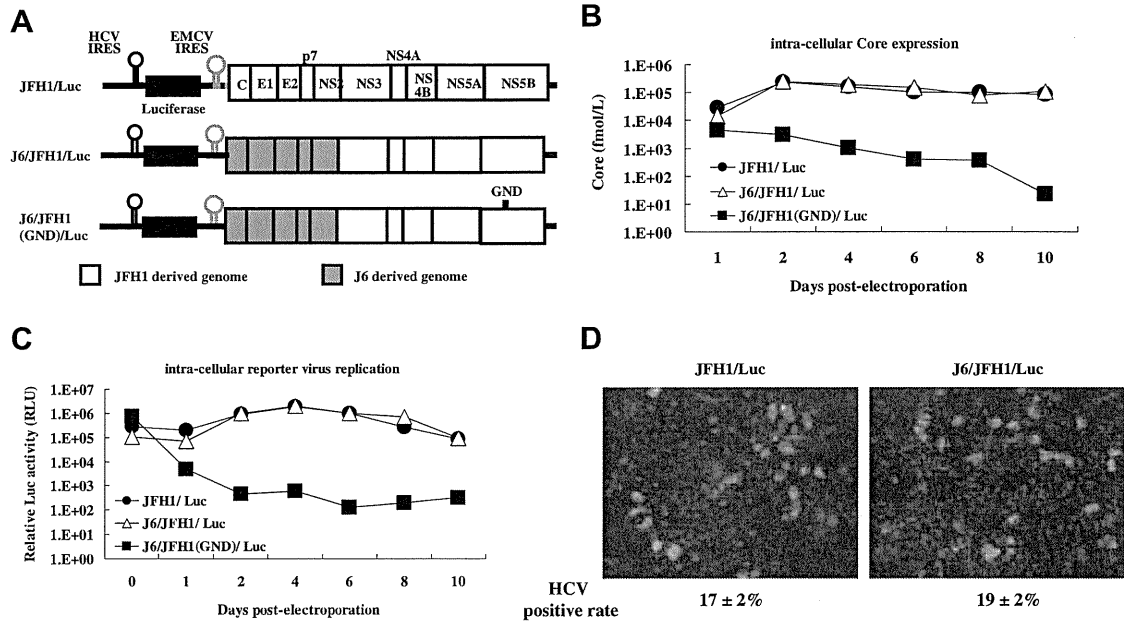


Fig. 1. Schematics of luciferase reporter HCV in this study. (A) Organization of luciferase reporter HCV. The luciferase gene is depicted as a black box. The JFH1-derived open reading frame and J6-derived open reading frame are depicted as a gray box and white box, respectively. As a negative control, a GND mutation was introduced to NS5B RdRp. (B, C) Virus replication kinetics in Huh7.5 cells of luciferase reporter HCV. The cells were electroporated with luciferase reporter RNA as described in Materials and methods, and the cells were assayed for core protein ELISA (B) and luciferase activity (C) at intervals as indicated. The assays were repeated at least three times, and the mean values are presented. Huh7.5 cells electroporated with JFH1/Luc or J6/JFH1/Luc RNA were subjected to indirect immunofluorescence analysis at 3 days post-electroporation (D). Cells were incubated with an HCV-infected patient's serum followed by FITC-labeled goat anti-human IgG (green). In parallel, the cells were stained with Hoechst 33342 to visualize the nuclei (blue). The HCV-positive rate was calculated by counting the number of HCV-positive cells among the total cells, and the data represent the means and SE of three independent experiments.

analyzed by ELISA. As shown in Fig. 2A, electroporation of both reporter viral RNAs with Huh7.5 cells released the HCV core protein into the culture supernatants. The levels of core protein released from both reporter HCV RNAs peaked at 6 days post-electroporation. The amount of core protein of the J6/JFH1/Luc supernatants was 2–4 fold greater than that of JFH1/Luc among all the time points tested. In parallel, to analyze the infectivity of progeny virions produced from reporter RNA-electroporated cells, these supernatants were used as inocula for naïve Huh7.5 cells. The cells inoculated

with these supernatants were harvested at 48 h post-inoculation, and the luciferase activity of the cell lysate was analyzed (Fig. 2B). These supernatants infected naïve Huh7.5 cells, and transduced luciferase activity in the cells. The infectious virus of both reporter HCVs was initially detected on day 2 and peaked on day 4 post-electroporation. However, the infectivity was decreased after day 6 post-electroporation. Furthermore, the infectivity of J6/JFH1/Luc supernatants was significantly higher than that of JFH1/Luc (approximately 10-fold). To compare the luciferase activity and the virus titer, we

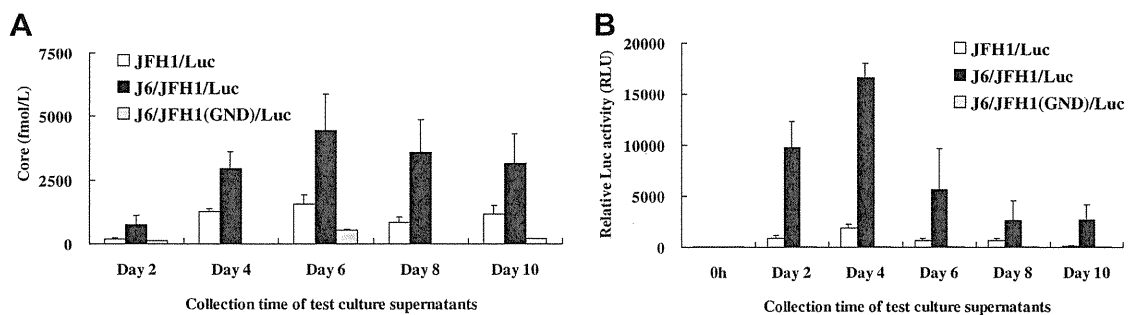


Fig. 2. Progeny virus production from luciferase reporter RNA-transfected Huh7.5 cells. The cells were electroporated with luciferase reporter RNA as described in Materials and methods, and culture supernatants of the cells were collected at the indicated time points. The amount of progeny virus in the supernatant was measured by the HCV core protein ELISA. (A) In parallel, the supernatants were added to naïve Huh7.5 cells. At 48 h post-addition, the cells were lysed, and assayed for luciferase activity to assess the infectivity of progeny virus from reporter HCV RNA. (B) The assays were repeated at least three times, and the mean values are presented.

performed standard virion titration by immunofluorescent antibody staining. The result showed that the virus titer of J6/JFH1/Luc supernatant, collected at day 4 post-RNA transfection, was 5×10^3 fluorescent-focus forming units (ffu) per ml. In contrast, the titer of JFH1/Luc supernatant was below the detection limit ($<1 \times 10^2$ ffu/ml). Interestingly, the peaks of the core release and the infectivity were slightly different, i.e., the peak of the core release of J6/JFH1 was on day 6, and that of the infectivity was on day 4 post-electroporation. Collectively, these data revealed that J6/JFH1 had a greater ability to release progeny virions than JFH1, though the levels of intracellular replication were comparable between J6/JFH1 and JFH1.

3.3. Characterization of cell-free infectious progeny virions in luciferase reporter HCV RNA-transfected cells

Next, we examined whether the J6/JFH1/Luc-derived supernatants had the features of a virus and thus could be used as a surrogate for HCV. The supernatants collected from each culture of reporter RNA-electroporated cells were irradiated with ultra-violet (UV) for 5 min, and the supernatants were then inoculated into naïve Huh7.5 cells. As shown in Fig. 3A, the infectivity of the reporter virus was completely abrogated by UV-irradiation. The results indicated that the luciferase activity transduced by the supernatants was derived from the genome of the reporter virus, not from incorporation of the luciferase protein into the virion. The entry of the HCV virion was mediated by binding between the cellular surface protein CD81 and the HCV envelope protein E2 [15]. Therefore, the naïve Huh7.5 cells were pre-treated with a recombinant monoclonal antibody against CD81. After 1 h pre-treatment, the J6/JFH1/Luc supernatant was inoculated into the cells, and the luciferase activity of cells was analyzed at 48 h post-inoculation (Fig. 3B). Normal mouse IgG showed no effect on the infectivity of the J6/JFH1/Luc supernatant. In contrast, the infectivity of the J6/JFH1/Luc supernatant was decreased by pre-treatment with anti-CD81 antibody in a dose-dependent manner. The results suggested that the supernatant from luciferase reporter J6/JFH1/Luc-transfected cells contained a virus with characteristics similar to HCV,

and that this reporter virus could be utilized to investigate all the steps of virus replication, including the intra-cellular viral replication, the virus production and the virus entry as a surrogate model of HCV.

3.4. Analysis of a potential role for ESCRT family proteins in HCV virus production

Prior to the recent establishment of the JFH1-based cell-culture system, there was no system for producing the HCV virus, and thus many aspects of the virus production of HCV still remain poorly understood. Generally, the production of the enveloped virus requires a multi-step process that includes the proper transport of viral proteins and organization of viral proteins on the cellular membrane, and these steps are coordinated by a variety of cellular factors [16,17]. From numerous intensive studies, it has been revealed that the process of budding of many enveloped viruses utilizes the ESCRT machinery, which is responsible for the formation of luminal vesicles of endosomal multivesicular bodies (MVB) [16,18–20]. The ESCRT machinery consists of a number of cellular proteins that make up three functional sub-complexes – ESCRT-I, ESCRT-II and ESCRT-III – and other related factors; i.e., Vps4 and AIP/Alix are also participated in the function of ESCRT machinery [20]. A series of analyses about ESCRT networks has revealed the consensus amino acid motifs of viral proteins; the P(T/S)AP motif was observed to interact with Tsg101, and the YPxL motif was seen in the case of AIP/Alix [19]. We searched for these motifs in the J6 and JFH1 genomes, and found one AIP/Alix interacting the YPxL motif in the NS5B region (aa. 2604 to 2607; YPDL). Therefore, the relation between ESCRT and HCV was examined by analyzing the virus production using a luciferase reporter HCV system. First, we constructed the expression plasmids of the ESCRT-I protein Tsg101, and the ESCRT-associated proteins Nedd4L and AIP/Alix. The ESCRT expression plasmids were transfected into the J6/JFH1/Luc or JFH1/Luc RNA-transfected Huh7.5 cells. After 48 h of transfection, the culture supernatants were collected and inoculated into the culture of the naïve Huh7.5 cells. The effects of over-expression of ESCRT proteins on intra-cellular virus

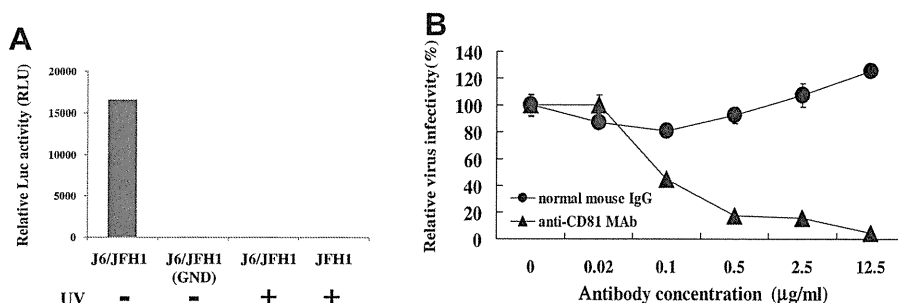


Fig. 3. Anti-CD81 antibody blocks luciferase reporter HCV infection. The reporter viruses containing supernatants were prepared as described in Materials and methods. (A) The JFH1/Luc and J6/JFH1/Luc supernatants were irradiated with UV at 5 min, and then added to naïve Huh7.5 cells. The infectivity was analyzed by luciferase assay. (B) Huh7.5 cells were pre-treated with anti-CD81 monoclonal antibody or control mouse IgG at 1 h before infection. Cells were then infected with J6/JFH1/Luc reporter viruses for 6 h. At 48 h post-infection, the cells were lysed and assayed for luciferase activity. Activities are expressed as the relative activity compared to that of the null antibody-treated sample. The assays were repeated at least three times, and the mean values are presented.

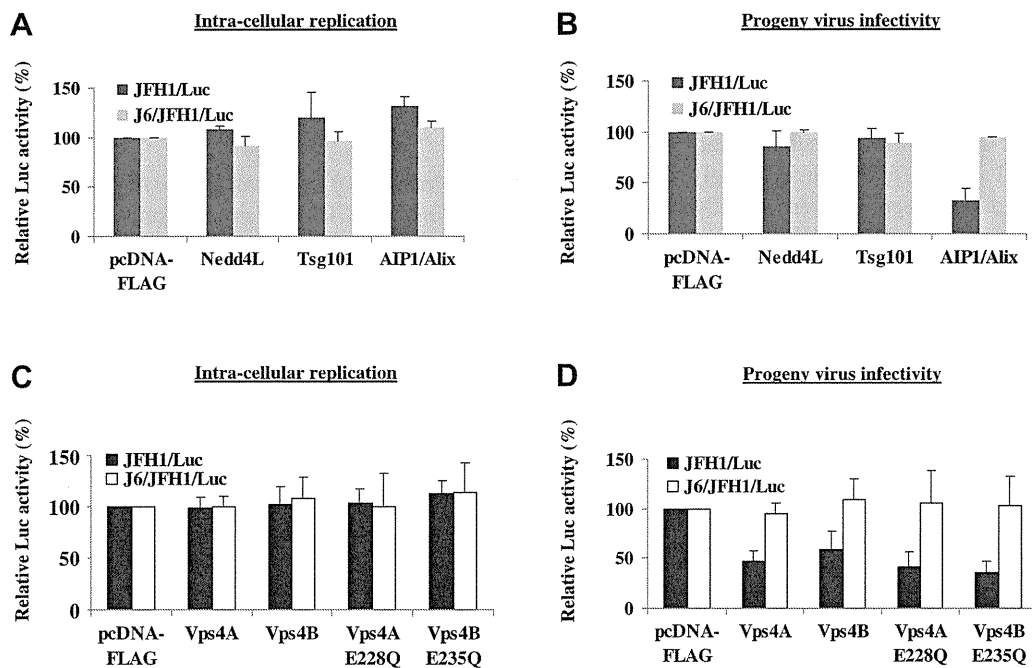


Fig. 4. Effect of ESCRT family protein expression on intra-cellular replication and progeny virus production in Huh7.5 cells. Huh7.5 cells were electroporated with JFH1/Luc and J6/JFH1/Luc RNA, respectively. The RNA-electroporated cells were then transfected with ESCRT protein expression plasmids. At 96 h after transfection, the culture supernatants were collected, and the cells were harvested. (A, C) Cell lysates were assayed for luciferase activity to assess intra-cellular virus replication. (B, D) Collected supernatants were added to naïve Huh7.5 cells and incubated for 48 h, and then the luciferase activity of the cells was analyzed to assess progeny virus infectivity. The data relative to that of luciferase activity in the absence of ESCRT protein (pcDNA-FLAG) is indicated. The assays were repeated at least three times, and the mean and standard error are presented.

replication and virus production were analyzed by monitoring the luciferase activity of reporter RNA-transfected cells (Fig. 4A), and the luciferase activity expressed by supernatant virus (Fig. 4B). As shown in Fig. 4A, the overexpression of Nedd4L, Tsg101, and AIP/Alix had no effect on the intra-cellular replication of either reporter HCV. As shown in Fig. 4B, the virus production from J6/JFH1/Luc also was not affected by these ESCRT protein expressions. In contrast, the expression of AIP/Alix decreased the virus production from JFH1/Luc by 50%. This result implied that the ESCRT machinery might have played some role in the difference in the efficacy of virus production observed between JFH1 and J6/JFH1. AAA-ATPase Vps4, which is present in humans in two isoforms (Vps4A and Vps4B), is a key modulator protein for the final step of ESCRT machinery. To analyze the role of ESCRT in HCV virus production, we constructed expression vectors for Vps4A and 4B, as well as expression vectors for a dominant-negative Vps4A(E228Q) and Vps4B(E235Q) [19]. As shown in Fig. 4C, the intra-cellular replications of JFH1 and J6/JFH1 were not influenced by the wild-type or dominant-negative Vps4 expression. In contrast, the levels of virus production of JFH1/Luc were reduced up to 50% by the expression of both dominant-negative Vps4 mutants (Fig. 4D). Interestingly, neither dominant-negative Vps4 influenced the virus production of J6/JFH1/Luc. These results implied that JFH1 might utilize the ESCRT machinery for release of infectious virus particles.

3.5. Effect of polyions on the infectivity of the J6/JFH1/Luc reporter virus

Next, we tested the usefulness of the J6/JFH1/luc reporter system for virus entry analysis. The binding of the viral and cellular receptors is coordinated with the ionic conditions, indicating that compounds that affect the ionic charge of the receptor surface might be potent inhibitors of virus infection [21,22]. Polyions with a positive or negative charge are frequently used for virus entry analyses, and exhibit inhibitory activity on virus infection [21,22]. Therefore, we investigated the effect of different polyions on the infectivity of the J6/JFH1/Luc virus in order to clarify the influence of electrostatic interactions in virus binding to cell membranes. As candidate compounds, we used both polymers having a positive charge (polybrene (size of 3000 Da), DEAE-dextran (100,000 Da), and poly-L-lysine (500,000 Da)) and those having a negative charge (heparin (15,000 Da), dextran sulfate (50,000 Da), and polyvinyl sulfate (150,000 Da)). These polymers were added to the Huh7.5 cells at 1 h before inoculation of the J6/JFH1/Luc virus into the cells. After 48 h of inoculation, the cells were harvested and the luciferase activity was analyzed (Fig. 5A and B). As shown in Fig. 5A, two polyanions, heparin and polyvinyl sulfate, decreased the infectivity of J6/JFH1/Luc virus in a dose-dependent manner, whereas one polyanion, dextran sulfate, enhanced the infectivity up to 2-fold. In the case of polycations, the addition of polybrene enhanced virus

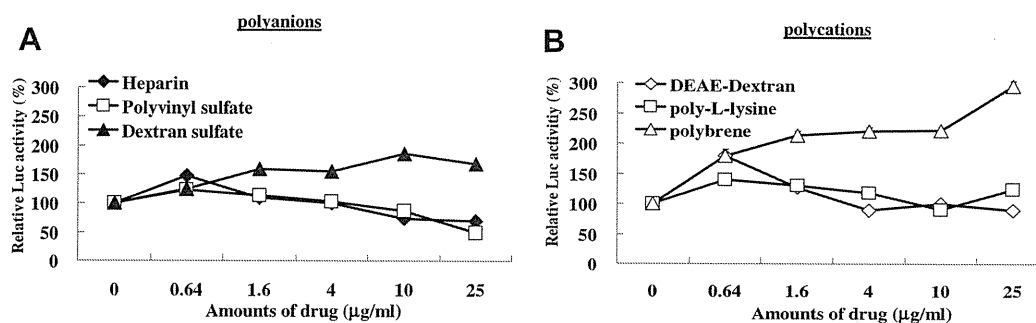


Fig. 5. Effect of multiple polyions on J6/JFH1/Luc virus infection of Huh7.5 cells. Huh7.5 cells were infected with J6/JFH1/Luc virus in the presence of each of the polyions for 6 h, and a luciferase assay was performed 48 h later. The data was expressed as the relative activity compared to the luciferase activity in the absence of polyions. The assays were repeated at least three times, and the mean values are presented.

infection up to 3-fold in a dose-dependent manner, although poly-L-lysine and DEAE-dextran showed no effect on the infectivity of the J6/JFH1/Luc virus (Fig. 5B). The effect shown by compounds belonging to positive and negative polyions suggested that the electric charge is not sufficient by itself to explain the inhibitory or enhancing activity of these drugs on the HCV virus entry. These results indicated that the J6/JFH1/Luc virus was useful to easily monitor HCV virus entry.

3.6. Screening of microRNA inhibition on intra-cellular HCV replication

To confirm the usefulness of the J6/JFH1/Luc reporter system in the analysis targeting intra-cellular replication of HCV, we analyzed the possible involvement of micro RNAs (miRNAs) in HCV infection. miRNAs are evolutionarily conserved, small, non-coding RNA molecules that regulate gene expression at the level of translation [23,24]. Recently, it has been reported that some miRNAs influence the replication of HCV in the cells [25–27]. For example, the expression of miR-122 in the cells might be essential for HCV replication [25]. In addition, the number of miRNAs has been increasing due to numerous strenuous analyses in recent years. Therefore, we compared the full sequences of the viral genome among 4

different HCV strains (H77C, Con1, J6, JFH1) with the sequences of 630 human miRNAs using the miRNA database program (RegRNA: <http://regna.mbc.nctu.edu.tw/index.php>), and then identified 54 miRNAs that matched with at least one HCV strain. 10 of the 54 miRNAs matched with all four HCV strains. Hence, we focused on analysis of the function of the 10 miRNAs on HCV replication and prepared commercially available miRNA inhibitors (Anti-miR™ miRNA inhibitor, Ambion) that were chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous target miRNA molecules. The J6/JFH1/Luc RNA-electroporated cells were transfected with each of the 10 specific miRNA inhibitors and the luciferase activities were analyzed at 48 h post-transfection of the inhibitors. None of the miRNA inhibitors significantly affected the cell viability (data not shown). As shown in Fig. 6A, the inhibition of miR-122 reduced the level of intra-cellular virus replication by up to 50% as previously reported [25]. A similar reduction of viral replication was also observed by treatment with the miR-34b inhibitor. The treatment with an anti-miR negative control that is a random sequence anti-miR molecules that has been extensively tested in human cell lines and validated to not produce identifiable effects on known miRNA functions showed no significant effect on HCV replication. None of the other inhibitors showed any significantly greater effect on the

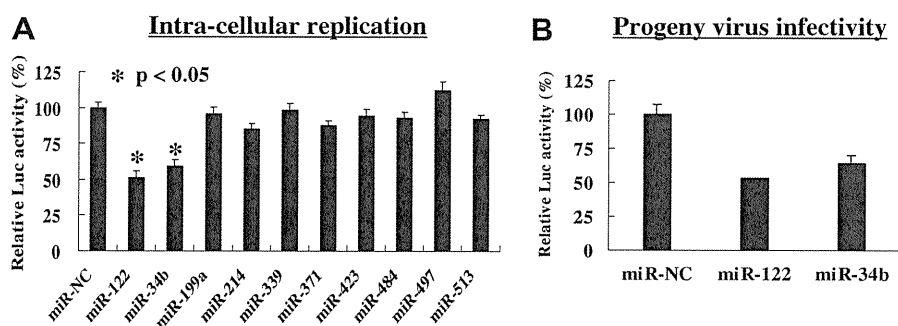


Fig. 6. Effect of miRNA inhibitor on intra-cellular replication of J6/JFH1/Luc RNA in Huh7.5 cells. Target cells were electroporated with J6/JFH1/Luc RNA, and then transfected with a miRNA-specific or a non-target negative control miRNA inhibitor. At 48 h post-transfection, cells were harvested and analyzed for luciferase activity (A). In parallel, the culture supernatants were collected at 48 h post-transfection to assess the effect of miRNA inhibitors on the virus production. The supernatants were added to naïve Huh7.5 cells, and the progeny virus infectivities were then analyzed by luciferase assay. (B) The data relative to the luciferase activity obtained from a non-target negative control miRNA inhibitor are indicated. The assays were repeated at least three times, and the mean and standard error are presented. Statistical significance relative to the negative control miRNA samples as calculated by *t*-test is shown (* $p < 0.05$).

virus replication than the anti-miR negative control. The miR-34b and miR-122 inhibitors decreased the virus production to the levels 64% and 53% of the control, respectively (Fig. 6B). Since the extent of the reduction in virus production was comparable with that of intra-cellular HCV RNA levels (Fig. 6A), it was likely that these miRNA inhibitors affected the intra-cellular viral replication rather than interfering with the particle formation and the release of the virion. These results suggest that the function of miR-34b could affect the replication of HCV, and also suggested that the J6/JFH1/Luc system was useful to analyze the intra-cellular replication of HCV.

4. Discussion

In this report, we generated two bicistronic luciferase reporter HCV clones from JFH1 and J6/JFH1, and established a unifying system that can monitor intra-cellular viral replication, virion production, and virus entry. Using two constructs, we initially compared the potential of intra-cellular viral replication and virus production. After transfection of reporter RNAs, the level of the intra-cellular core protein and the luciferase activity in RNA-transfected cells showed similar kinetics for JFH1/Luc and J6/JFH1/Luc (Fig. 1B and C). In contrast, both the efficacy of core protein production into the culture supernatant and the infectivity of supernatant virus from J6/JFH1/Luc were significantly higher than that of JFH1/Luc (Fig. 2A and B). These results indicated two possibilities that JFH1 and J6/JFH1 utilize different machinery for progeny virus packaging and budding, or that they utilize the same machinery for the virus production but to a different degree. To evaluate the difference in the virus production between JFH1 and J6/JFH1, we analyzed the role of ESCRT machinery in virus production (Fig. 4A–D). Dominant-negative Vps4 expression inhibited JFH1/Luc virus production, but did not influence J6/JFH1/Luc virus production. In the course of preparing this manuscript, Corless et al. reported that HCV requires late components of the ESCRT pathway for release of infectious virus particles [28]. They showed that a dominant-negative Vps4 expression inhibited the production of virus-like particles derived from JFH1 in a dose-dependent manner. The findings reported by Corless et al. and the findings of our present study emphasize that the ESCRT machinery plays an essential role in JFH1 virus production.

To examine the virus entry, we analyzed the effect of anti-CD81 antibody and polyions on reporter virus infectivity (Figs. 3B and 5A and B). The pre-treatment with anti-CD81 antibody decreased the infectivity of the reporter J6/JFH1 virus in a dose-dependent manner. The result suggested that the reporter J6/JFH1 virus, similar to HCVcc, utilized the CD81 as a major entry receptor, and that our reporter virus could be used as a surrogate model of HCV entry analysis. As a result of polyions analysis, one of the polycations (dextran sulfate) and one of the polyanions (polybrene) increased the reporter virus infectivity, and the remainder of the polyions inhibited the virus infectivity. These results indicate the

possibility that not only the electrostatic condition of polyions but also their molecular weight may be a determinant of the receptor binding of HCV. Considering that several membrane molecules have been identified as candidate cellular receptors for HCV entry [15,29,30], the polyions could interact with a different molecule(s) to influence virus production. As for heparin, it was reported that cell surface heparan sulfate proteoglycans play an important role in mediating HCV envelope–target cell interaction [31]. Basu et al. [32] also reported that heparin treatment completely blocked HIV/HCV E1–E2 pseudotype infection. In their analysis, however, the inhibitory effect of heparin against cell culture-grown HCV H77 was somewhat lower than that of HIV/HCV E1–E2 pseudotypes. In our present study, the level of inhibitory effect of heparin on J6/JFH1 reporter virus infection was not so prominent. Collectively, these data suggest a possibility that cell surface heparan sulfate proteoglycans contribute to the infection of both HIV/HCV E1–E2 pseudotype and cell culture-grown HCV with a different degree. Therefore, to develop a polyion-based anti-HCV drug, a more detailed assessment of the interaction between each candidate receptor and polyion is necessary.

Using microRNA inhibitors, the decrease of miR-34b expression suppressed intra-cellular HCV replication (Fig. 6A). miR-34b belongs to the evolutionary conserved microRNA family of miR-34s [33], known for their role in the p53 tumor suppressor network [34]. miR-34s have been shown to be controlled in a tissue-specific manner by p53. Both wild-type and mutant-type p53 protein expressions in serum and cytoplasm of liver tissue were more pronounced in patients with hepatocellular carcinoma associated with HCV infection [35]. Wild-type p53 binds to a transcriptional regulatory element of miR-34s, thereby up-regulating miR-34 expression [34]. However, it is not understood whether the mutant-type p53 increases miR-34b expression. Furthermore, HCV replication in chronic hepatitis is higher than that of hepatocellular carcinoma [36]. Therefore, more detailed research is needed to reveal the significance of miR-34b expression in HCV replication and hepatocellular carcinoma.

As mentioned above, we have generated a recombinant luciferase reporter HCV, and have shown that the reporter HCV could be used for the quantitative analyses of intra-cellular replication, virus entry, and virion production. In general, the intra-cellular HCV replication has been analyzed by the quantitative real-time RT-PCR method that could detect a small amount of viral RNA because of the greatly high sensitivity. However, the real-time RT-PCR method involves multi-step procedures of the RNA extraction, the reverse transcription and the PCR reaction, which require skillfulness to perform. The high sensitivity and the multiple-steps of the real-time RT-PCR system sometimes cause an experimental error(s) when conducted by less-experienced individuals. On the other hand, our HCV luciferase reporter system is simpler and easier to perform compared to the real-time PCR system. The significant advantage of the reporter HCV is that it can analyze a large number of samples at a time in a time- and cost-saving manner. Also, it can be used to evaluate all the

events of viral life cycle. By using it, we have started the screening of anti-HCV substances from the natural resource chemical libraries and found a number of potential candidates for the analysis. Thus, this system can be applicable for robust screening analyses of chemical compounds to discover a potential therapeutic target of HCV.

Acknowledgments

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Sequence Heterogeneity in NS5A of Hepatitis C Virus Genotypes 2a and 2b and Clinical Outcome of Pegylated-Interferon/Ribavirin Therapy

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Abstract

Pegylated-interferon plus ribavirin (PEG-IFN/RBV) therapy is a current standard treatment for chronic hepatitis C. We previously reported that the viral sequence heterogeneity of part of NS5A, referred to as the IFN/RBV resistance-determining region (IRRDR), and a mutation at position 70 of the core protein of hepatitis C virus genotype 1b (HCV-1b) are significantly correlated with the outcome of PEG-IFN/RBV treatment. Here, we aimed to investigate the impact of viral genetic variations within the NS5A and core regions of other genotypes, HCV-2a and HCV-2b, on PEG-IFN/RBV treatment outcome. Pretreatment sequences of NS5A and core regions were analyzed in 112 patients infected with HCV-2a or HCV-2b, who were treated with PEG-IFN/RBV for 24 weeks and followed up for another 24 weeks. The results demonstrated that HCV-2a isolates with 4 or more mutations in IRRDR (IRRDR[2a]≥4) was significantly associated with rapid virological response at week 4 (RVR) and sustained virological response (SVR). Also, another region of NS5A that corresponds to part of the IFN sensitivity-determining region (ISDR) plus its carboxy-flanking region, which we referred to as ISDR+C[2a], was significantly associated with SVR in patients infected with HCV-2a. Multivariate analysis revealed that IRRDR[2a]≥4 was the only independent predictive factor for SVR. As for HCV-2b infection, an N-terminal half of IRRDR having two or more mutations (IRRDR[2b]/N≥2) was significantly associated with RVR, but not with SVR. No significant correlation was observed between core protein polymorphism and PEG-IFN/RBV treatment outcome in HCV-2a or HCV-2b infection. **Conclusion:** The present results suggest that sequence heterogeneity of NS5A of HCV-2a (IRRDR[2a]≥4 and ISDR+C[2a]), and that of HCV-2b (IRRDR[2b]/N≥2) to a lesser extent, is involved in determining the viral sensitivity to PEG-IFN/RBV therapy.

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease, such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, with 180 million people being currently infected with HCV worldwide. It is estimated that 70% of acute infections become persistent [1]. As a consequence of the long-term persistence of HCV infection, the number of patients with hepatocellular carcinoma is expected to increase further over the next 20 years. More than two decades have passed since the discovery of HCV, and yet therapeutic options remain limited. Standard regimens for treatment of chronic hepatitis C include pegylated interferon alpha (PEG-IFN) and ribavirin (RBV) [2]. In addition, two protease inhibitors (telaprevir and boceprevir) were approved in May 2011 by the U. S. Food and Drug Administration (FDA) for clinical use in combination with PEG-IFN/RBV to treat chronic hepatitis C patients with HCV genotype 1 [3,4].

In Japan, about 70% of HCV-infected patients are infected with HCV genotype 1b (HCV-1b) and most of the remaining patients are infected with HCV-2a (25%) or HCV-2b (5%) [5]. When treated with PEG-IFN/RBV, the sustained virological response (SVR) rate is ca. 50% in HCV-1b infection, and ca. 80% in HCV-2a and -2b infections [2,6]. The mechanism(s) underlying the different responses among patients with different HCV genotypes and subtypes is still unclear. However, this suggests that viral genetic heterogeneity could affect, at least to some extent, the sensitivity to IFN-based therapy. In this context, sequence heterogeneity of the viral NS5A protein has been widely discussed for its correlation with IFN responsiveness. Sequence variations within a region in NS5A of HCV-1b defined as the IFN sensitivity-determining region (ISDR) is correlated with IFN responsiveness [7]. In HCV-2a infection, the influence of sequence heterogeneity in and around a region corresponding to ISDR on the IFN responsiveness was also suggested [8–10]. Recently, we identified a

new region near the C-terminus of NS5A of HCV-1b, which we refer to as the IFN/RBV resistance-determining region (IRRDR) [11,12]. The degree of sequence variation within IRRDR was significantly correlated with the clinical outcome of PEG-IFN/RBV combination therapy. The significance of IRRDR of other HCV genotypes, however, has not been investigated yet.

In addition to the NS5A sequence variation, HCV core protein polymorphism was also proposed as a pretreatment predictor of poor virological response in HCV-1b-infected patients treated with PEG-IFN/RBV therapy [13]. It is not clear at this stage whether core protein polymorphism could be used to predict the treatment outcome in HCV-2a and -2b infections. In the present study, we investigated the impact of viral genetic heterogeneity in the NS5A and core regions of HCV-2a and -2b on PEG-IFN/RBV treatment outcome. To the best of our knowledge, this is the first report describing the possible correlation between PEG-IFN/RBV responsiveness and NS5A-IRRDR heterogeneity of HCV-2a and -2b.

Materials and Methods

Ethics statement

The study protocol, which conforms to the provisions of the Declaration of Helsinki, was approved beforehand by the Ethic Committees in Kobe Asahi Hospital and Kobe University, and written informed consent was obtained from each patient prior to the treatment.

Patients

A total of 112 patients seen at Kobe Asahi Hospital and Kobe University Hospital, Kobe, Japan, who were chronically infected with HCV-2a (61 patients) or HCV-2b (51 patients), were enrolled in the study. HCV subtype was determined according to the method of Okamoto et al. [14]. The patients were treated with PEG-IFN α -2b (Pegintron[®]; Schering-Plough, Kenilworth, NJ) (1.5 μ g per kilogram body weight, once weekly, subcutaneously) and RBV (Rebetol[®]; Schering-Plough) (600~800 mg daily, per os), for 24 weeks according to a standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labour and Welfare, Japan. All patients received >80% of scheduled dosage of PEG-IFN and RBV. Serum samples were collected from the patients at intervals of 4 weeks before, during and after the treatment, and tested for HCV RNA and core antigen titers as reported previously [15].

Sequence analysis of the NS5A and core regions

HCV RNA was extracted from 140 μ l of serum using a commercially available kit (QIAmp viral RNA kit; QIAGEN, Tokyo, Japan). The extracted RNA was reverse transcribed and amplified for NS5A and core regions using Super script III one step RT-PCR platinum Taq HiFi (Invitrogen, Tokyo, Japan). The resultant RT-PCR product was subjected to a second-round PCR by using Platinum Taq DNA polymerase high fidelity III (Invitrogen). Primers used for amplification of full-length NS5A of the HCV-2a and -2b genomes and those of the core region of HCV-2a were reported previously [16,17]. Primers for amplification of the core region of HCV-2b are as follows: C-2b/1 (5'-AGCCATAGTGGTCTGCGGAACC-3'; sense, nucleotides [nt] 136 to 157) and C-2b/4 (5'-GGAACARTTGCACTCTTGGGTG-3'; antisense, nt 1241 to 1262) for one step RT-PCR; C-2b/2 (5'-CCACTCTATGTCCGGTCATTTGG-3'; sense, nt 208 to 230) and C-2b/3 (5'-GAGCTGCCAGGTGATGCTG-3'; antisense, nt 971 to 989) for the second round PCR. RT was performed at 45°C for 30 min and terminated at 94°C for 2 min,

followed by the first-round PCR over 35 cycles, with each cycle consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 68°C for 90 sec. The second-round PCR was performed under the same condition. The sequences of the amplified fragments were determined by direct sequencing without subcloning. The amino acid (aa) sequences were deduced and aligned using GENETYX Win software version 7.0 (GENETYX Corp., Tokyo, Japan). The numbering of aa residues for HCV-2a and -2b isolates is according to the polyprotein of HCV-J6 [18] and -J8 [19], respectively.

Statistical analysis

Numerical data were analyzed by Student's *t* test while categorical data by Fisher's exact probability test [8]. To evaluate the optimal threshold of the number of aa mutations in ISDR and IRRDR for prediction of treatment outcomes, the receiver operating characteristic curve was constructed. Univariate and multivariate logistic regression analyses were performed to identify independent predictors for treatment outcomes. All statistical analyses were performed using the SPSS version 16 software (SPSS Inc., Chicago, IL). Unless otherwise stated, a *P* value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers

The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB600751 through AB600834.

Results

Patients' Responses to PEG-IFN/RBV Combination Therapy in HCV-2a and HCV-2b infections

Of the 61 patients infected with HCV-2a, 46 (75%) patients cleared HCV viremia by week 4 (rapid virological response [RVR]), and all the patients (100%) by week 12 (early virological response [EVR]) and at week 24 (end-of-treatment response [ETR]) (Table 1). Likewise, of 51 patients infected with HCV-2b, 34 (67%), 51 (100%) and 50 (98%) patients achieved RVR, EVR and ETR, respectively. After the end of treatment, 105 patients (58 with HCV-2a and 47 with HCV-2b) could be followed up for another 24 weeks. At the end, SVR was achieved by 49 (84%) patients infected with HCV-2a and by 34 (72%) patients with HCV-2b. Only 9 (16%) and 13 (28%) patients with HCV-2a and -2b, respectively, were non-SVR. There was no case of null-response (continuous viremia throughout the treatment and follow up periods) since all the non-SVR patients once cleared viremia at a certain time point followed by a rebound in viremia either before or after the end of the treatment (relapse).

Comparison of the base line demographic characteristics between SVR and non-SVR patients revealed that, in HCV-2a infection, SVR patients had a significantly lower average age than that of non-SVR (Table 2). In HCV-2b infection, on the other hand, SVR patients had significantly γ -GTP levels than those of non-SVR. There was no significant difference in viremia titers between SVR and non-SVR in patients infected with HCV-2a or -2b.

Sequence Analysis of NS5A of HCV-2a and HCV-2b

The entire NS5A region of the HCV-2a and -2b genomes in pretreatment sera were sequenced, and aa sequences deduced. All the sequences obtained were aligned and the consensus sequences for HCV-2a and -2b were inferred. An N-terminal half (aa 1977 to 2196) of the consensus sequences of HCV-2a and -2b isolates were each identical to the prototype sequences, HCV-J6 [18] and

Table 1. Proportions of various virological responses of HCV-2a- and HCV-2b-infected patients treated with PEG-IFN/RBV.

Response	Proportion		
	HCV-2a	HCV-2b	All
RVR	46/61* (75%)	34/51 (67%)	80/112 (71%)
Non-RVR	15/61 (25%)	17/51 (33%)	32/112 (29%)
EVR	61/61 (100%)	51/51 (100%)	112/112 (100%)
ETR	61/61 (100%)	50/51 (98%)	111/112 (99%)
SVR	49/58 (84%)	34/47 (72%)	83/105 (79%)
Non-SVR	9/58 (16%)	13/47 (28%)	22/105 (21%)

*No. of patients/no. of total.

Abbreviations: RVR, rapid virological response; EVR, early virological response; ETR, end-of-treatment response; SVR, sustained virological response.

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HCV-J8 [19], respectively. The remaining C-terminal half (aa 2197 to 2442) of the consensus sequences were identical to those reported by Murakami et al. [8] except that His at position 2358 in the HCV-2b sequence was replaced with Cys, which was more conserved (59% of the isolates tested) than His (22%).

To investigate the impact of NS5A heterogeneity on the clinical outcome of PEG-IFN/RBV therapy, we first performed a sliding window analysis with a window size of 20 residues over the full-length NS5A sequences obtained from 23 RVR and 7 non-RVR patients infected with HCV-2a along with the consensus sequence, as described previously [8]. This analysis revealed that the number of aa mutations differed significantly between RVR and non-RVR isolates in two regions within the C-terminal half of NS5A (data not shown). The more C-terminally located one exactly matched the region that corresponded to IRRDR of HCV-1b, ranging from aa 2332 to 2387, thus being referred to as IRRDR[2a] (see Figure 1). The other region composed of a part of ISDR plus its carboxy-flanking region, ranging from aa 2232 to 2262, thus being referred to as ISDR/+C[2a] (see Figure 2). It was confirmed that the average numbers of aa mutations in IRRDR[2a] and ISDR/+C[2a] were each significantly larger in isolates from RVR than those from non-RVR patients (Table 3). More importantly, the average numbers of aa mutations in IRRDR[2a] and ISDR/+C[2a] were each significantly larger in SVR than in non-SVR.

Sequences of IRRDR[2a] and ISDR/+C[2a] obtained from SVR and non-SVR patients and the number of mutations of each isolate are shown in Figures 1 and 2.

Likewise, a sliding window analysis on HCV-2b isolates (16 RVR and 6 non-RVR) identified an N-terminal part of IRRDR (aa 2332 to 2357), referred to as IRRDR/N[2b], that showed a significant difference in the number of aa mutations between RVR and non-RVR (data not shown). The average numbers of aa mutations in IRRDR/N[2b] were significantly larger in RVR than in non-RVR (Table 3). However, they did not differ significantly between SVR and non-SVR. Sequences of IRRDR[2b]/N obtained from RVR and non-RVR patients are shown in Figure 3.

Correlation between NS5A Sequence Heterogeneity and SVR or RVR in HCV-2a and HCV-2b infections

The receiver operating characteristic analysis identified the optimal thresholds of the numbers of aa mutations in IRRDR[2a] and ISDR/+C[2a] for the prediction of RVR and SVR in HCV-2a infection; four and one for IRRDR[2a] and ISDR/+C[2a], respectively (data not shown). Accordingly, we found that 86% (42/49) of SVR patients, and only 22% (2/9) of non-SVR, were infected with HCV-2a isolates having IRRDR with 4 or more mutations ($IRRDR[2a] \geq 4$) (Table 4). On the other hand, 14% (7/49) of SVR, and 78% (7/9) of non-SVR patients, were infected with isolates having IRRDR with 3 or less mutations ($IRRDR[2a] \leq 3$). These results suggested that $IRRDR[2a] \geq 4$ was significantly associated with SVR ($P=0.0003$). Similarly, 93% (42/46) of RVR patients, and only 33% (5/15) of non-RVR, were infected with HCV-2a isolates of $IRRDR[2a] \geq 4$ while 7% (4/46) of RVR patients, and 67% (10/15) of non-RVR, were infected with HCV-2a isolates of $IRRDR[2a] \leq 3$, with the results suggesting that $IRRDR[2a] \geq 4$ was significantly associated with RVR as well ($P<0.0001$).

As for ISDR/+C[2a] heterogeneity, 71% (35/49) of SVR, and 22% (2/9) of the non-SVR patients, were infected with HCV-2a isolates with ISDR/+C having one or more mutation ($ISDR/+C[2a] \geq 1$) (Table 4). On the other hand, 29% (14/49) of SVR patients, and 78% (7/9) of the non-SVR, were infected with isolates with ISDR/+C without mutation ($ISDR/+C[2a] = 0$). Thus, $ISDR/+C[2a] \geq 1$ was significantly associated with SVR ($P=0.008$).

Table 2. Demographic characteristics of HCV-2a- and HCV-2b-infected patients with SVR and non-SVR.

Factor	HCV-2a			HCV-2b		
	SVR	Non-SVR	P value	SVR	Non-SVR	P value
Age	49.78±13.67*	62.89±7.01	0.007	50.03±15.03	55.08±11.22	0.28
Sex (male/female)	22/27	3/6	0.72	17/17	8/5	0.53
Body weight (kg)	60.39±11.00	54.67±10.51	0.15	57.72±13.46	65.08±7.26	0.06
Platelets ($\times 10^4/\text{mm}^3$)	18.54±5.71	19.43±10.78	0.72	17.57±5.65	15.20±7.281	0.27
Hemoglobin (g/dl)	14.38±6.07	14.0±1.56	0.88	14.19±1.59	13.78±1.5	0.49
γ -GTP (IU/L)	37.66±53.25	36.83±24.82	0.97	39.68±34.33	81.30±69.11	0.02
ALT (IU/L)	64.75±52.45	94.38±141.3	0.28	86.35±91.95	86.85±118.7	0.98
HCV-RNA (KIU/ml)	1350±1424	1598±1464	0.63	5543±7643	7905±14210	0.47
HCV core antigen (fmol/L)	6543±6927	6105±8290	0.91	9054±6743	9390±8723	0.92

*Mean ± S.D.

Abbreviations: SVR, sustained virological response; γ -GTP, gamma glutamyl transpeptidase; ALT, alanine aminotransferase.

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